Oregano Flavonoids as Lipid Antioxidants

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Oregano (*Origanum vulgare* L.) leaves were successively extracted with hexane, ethyl ether, ethyl acetate and ethanol. The ethanol extract was reextracted in a separatory funnel with petroleum ether, ethyl ether, ethyl acetate and butanol. The ethyl ether layer was the most effective in stabilizing lard against oxidation, with activity equal to butylated hydroxytoluene. It also showed antioxidant activity when tested on vegetable oils under storage or frying conditions. The main antioxidant factors isolated from the ethyl ether layer consisted of flavonoids. Chromatographic and spectrophotometric analysis demonstrated the presence of the flavone apigenin, the flavanone, eriodictyol and the dihydroflavonols, dihydrokaempferol and dihydroquercetin.

KEY WORDS: Antioxidant activity of flavonoids, antioxidants, flavonoids, lipid oxidation, natural antioxidants, oregano extracts, oregano flavonoids, plant extracts.

Flavonoids are widely distributed in the plant kingdom. Several relevant compounds have been isolated from plant extracts and found to possess antioxidant activity (1–4). The efficiency of polyhydroxyflavonoids in relation to their structure also has been investigated (5–7).

Oregano extracts have shown a pronounced effect in stabilizing lipids against autoxidation (8,9). In a recent study we extracted oregano leaves with solvents of increasing polarity, namely hexane, ethyl ether (EE), ethyl acetate (EA) and ethanol, and found all the extracts effective in stabilizing lard. We were able to isolate the main antioxidant factors of the hexane extract (10). The present work was undertaken to isolate and identify the antioxidant compounds of the ethanol extract of oregano leaves.

EXPERIMENTAL PROCEDURES

Preparation of the extracts. Oregano leaves (200 g) were successively extracted with hexane, ethyl ether, ethyl acetate and 95% ethanol as described by Vekiari *et al.* (10). The ethanol extract was further processed for the isolation of antioxidants as follows: the extract was filtered, and the filtrate was dried under vacuum at 40°C in a rotary evaporator. The residue was thoroughly mixed with 400 g boiling water and filtered. The filtrate was successively extracted in a separatory funnel with petroleum ether, b.p. 40–60°C (PE), EE, EA and butanol. Several 100-mL portions of each solvent were used until the solvent layer was colorless. The obtained layers were dried with anhydrous Na_2SO_4, filtered, partially concentrated and kept in sealed dark bottles until use.

Column chromatography. The EE layer was further fractionated by column chromatography. A glass column (i.d. 2.5 cm, length 50 cm) was used, packed with silica gel (7734 Merck, Darmstadt, Germany). Six fractions were obtained by stepwise gradient elution with 1, 5, 10, 50 and 100% methanol in dichloromethane, as presented in Figure 1. The fraction Fr2 was refractionated on the same column by stepwise gradient elution with 5, 10 and 20% methanol in dichloromethane, to three subfractions, namely 2A, 2B and 2C (Fig. 1).

Thin-layer chromatography (TLC). TLC plates precoated with silica gel (5715 Merck) were used to separate the constituents of the subfraction 2B. The plates were developed with chloroform/acetone/formic acid (9:2:1, vol/vol/vol) (CAF). Bands of interest were scraped from TLC plates, soaked overnight in 50 mL methanol, filtered, evaporated in vacuum to near dryness in a rotary evaporator at 40°C and redissolved in 1 mL methanol. Further separation was conducted on polyamide sheets ($11F_{254}$ Merck) with benzene/methyl ethyl ketone/methanol (4:3:3, vol/vol/vol) (BMM) as solvent. The eluted components were further separated by two-dimensional technique on cellulose plates (5716 Merck). Chromatograms were developed in the upper phase of *n*-butanol/acetic acid/water (4:1:5, vol/vol/vol) (BAW), air dried and redeveloped in the second dimension in 15% acetic acid. Other developing systems used were ethyl acetate/acetic acid/water (8:2:4, vol/vol/vol)

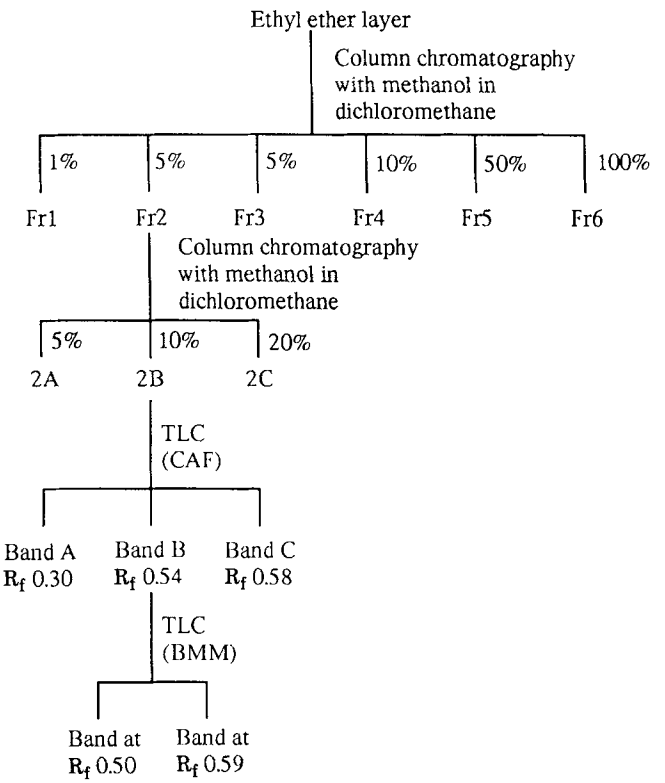

FIG. 1. Isolation of antioxidants from the ethyl ether layer. Percent refers to % methanol in dichloromethanol. Fr, fraction; TLC, thin-layer chromatography; CAF, chloroform/acetone/formic acid; BMM, benzene/methyl ethyl ketone/methanol.

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(EAW) on cellulose plates and methanol/acetic acid/water (72:4:4, vol/vol/vol) (MAW) on polyamide sheets.

The following spray reagents were used to identify chemical compounds (i) aluminum chloride 5% solution in water. A yellow color indicates the presence of flavonoids (11,12); (ii) naturstoffreagent 2-amino-ethylester-diphenylboric acid (0.75% in methanol). Flavonoids present characteristic fluorescences when sprayed with naturstoff-reagent. A yellow-green fluorescence is indicative of 4′-OH flavonoids (12). Especially kaempferol derivatives have a bright, yellow-green color (13). Orange-yellow fluorescence indicates the presence of quercetin derivatives (13).

Tests of antioxidant activity. Storage tests were conducted on lard and refined corn oil, soybean oil and olive residue extracted oil. The oils were obtained from a commercial refining plant and contained no additives. Lard was melted at 85°C and filtered before use. A calculated quantity of the antioxidant (to obtain a concentration of 0.01 or 0.02%, w/w, on a dry basis) was added to 25 g of the substrate and dissolved by stirring. Comparative experiments were conducted with the commercial antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) or tertiary butylhydroquinone (TBHQ) at the same concentration as the oregano antioxidants. Control samples with no additives were prepared under the same conditions. The samples were incubated in the dark in 50-mL open beakers at 35 and 65°C. All experiments were run in duplicate and the presented results are the average of two trials. Significance of treatments was estimated by the Student's *t*-test at the probability level of 0.05 (14). At definite time intervals the peroxide values were determined according to Official Method Cd 8-53 of the American Oil Chemists' Society (15).

The EE layer was tested at frying temperature (180°C) in refined olive residue extracted oil at a concentration of 0.05%. TBHQ at the same concentration was tested for comparison. The samples (50 g) were heated in 100-mL open beakers on electric hot plates for 8-h periods daily, followed by overnight cooling at room temperature. The temperature was adjusted at 180 ± 3°C by a rheostat. All experiments were run in duplicate and the presented results are mean values of two trials. Samples were withdrawn and analyzed at definite time intervals. The absorbance at 232 nm (E_{232}), the iodine value (IV) and the refractive index (RI) were determined according to Standard Methods 2.505, 2.205 and 2.102, respectively, of the International Union of Pure and Applied Chemistry (16). Analysis of fatty acid composition was conducted on the starting oil and after nine days of frying by Official Method Ce 1-62 of the American Oil Chemists' Society (15). A Hewlett-Packard series 5700 gas chromatograph (Hewlett-Packard Co., Avondale, PA) was used, equipped with a flame-ionization detector and a packed column (15% DEGS, Chrom Pack, Middelburg, The Netherlands). The temperature of the injector and detector was adjusted to 250°C and the temperature of the oven to 180°C.

The bands separated by TLC were tested for antioxidant activity according to the carotene spray procedure of Philip as described by Pratt and Miller (4). β-Carotene (9 mg) was dissolved in 30 mL chloroform. Two drops of purified linoleic acid and 60 mL ethanol were added to the β-carotene-chloroform solution. The solution was sprayed on chromatograms streaked with the antioxidant solution, and the chromatograms were exposed to daylight until background color was bleached. Bands in which yellow color persisted possessed antioxidant activity.

Spectral identification of flavonoids. Spectra, 200–450 nm, were obtained by using a Varian DMS 80 ultraviolet (UV)-VIS spectrophotometer (Varian, Springvale, Australia). A solution of the antioxidant (3 mL at a concentration of 100 ppm) in methanol (spectral grade) was used as the test solution. The sodium methoxide spectrum was measured immediately after three drops of stock solution (2.5 g sodium dissolved in 100 mL methanol) were added to the test solution (12,17).

For the sodium acetate spectrum, coarsely powdered anhydrous sodium acetate was added to a cuvette containing the antioxidant solution until a 2-mm layer of sodium acetate remained at the bottom. The spectrum was recorded two minutes after the addition of the sodium acetate. The boric acid-sodium acetate spectrum was recorded after mixing with the addition of boric acid to the cuvette containing the sodium acetate-antioxidant solution (12,17).

The aluminum chloride spectrum was recorded immediately after the addition of six drops of aluminum chloride stock solution (5.0 g aluminum chloride dissolved in 100 mL methanol) to a cuvette containing the antioxidant solution. Three drops of hydrochloric acid-aluminum chloride stock solution (5.0 mL hydrochloric acid and 5.0 mL aluminum chloride solution) then were added to the cuvette, and the hydrochloric acid-aluminum chloride spectrum was recorded immediately (12,17).

RESULTS AND DISCUSSION

Antioxidant activity of the extracts. Table 1 presents the antioxidant activity of the ethanol extract and the obtained layers in lard, compared to BHA and BHT. The PE layer was too dilute (total solids content 0.02%, wt/vol) and was not tested. The EE layer was the most effective, with activity equal to BHT and much greater than the parent ethanol extract.

The results of the addition of the EE layer to vegetable oils stored at 65 and 35°C are given in Tables 2 and 3, respectively. The stability of the oils toward autoxidation was considerably increased with the addition of EE. Compared to BHT, the EE layer was a better antioxidant in most cases, but it was not as effective as TBHQ at 35°C.

TABLE 1

Antioxidant Activity of the Layers Obtained from the Ethanol Extract Tested on Lard in the Dark at 65°C (concentration 0.02%)[a]

	Peroxide value (meq/kg) after days						
Additive	0	2	6	9	13	17	20
None	2^b	9^c	95^e				
Ethanol extract	2^b	7^c	37^d				
Ethyl ether layer	2^b	3^b	6^b	11^b	12^b	15^b	21^b
Ethyl acetate layer	2^b	$4^{b,c}$	8^b	11^b	14^b	18^b	35^c
Butanol layer	2^b	$5^{b,c}$	15^c	80^d			
Butylated hydroxyanisole	2^b	$5^{b,c}$	15^c	34^c			
Butylated hydroxytoluene	2^b	$4^{b,c}$	7^b	10^b	12^b	19^b	21^b

[a]Values within a column followed by different superscript letters are significantly different ($P < 0.05$).

TABLE 2

Antioxidant Activity of the Ethyl Ether (EE) Layer Added to Vegetable Oils Stored in the Dark at 65°C (concentration 0.02%)[a]

Sample	Peroxide value (meq/kg) after days				
	0	3	6	9	13
Corn oil	3^{d}	11^{f}	71^{k}	166^{i}	
Corn oil + BHT[b]	3^{d}	12^{f}	50^{j}	127^{h}	
Corn oil + EE layer	3^{d}	7^{e}	25^{g}	64^{g}	
Soybean oil	1^{d}	$4^{d,e}$	38^{i}	126^{h}	
Soybean oil + BHT	1^{d}	3^{d}	$18^{e,f}$	69^{g}	
Soybean oil + EE layer	1^{d}	3^{d}	20^{f}	70^{g}	
Olive r. extd oil[c]	3^{d}	$10^{e,f}$	31^{h}	44^{f}	104^{f}
Olive r. extd oil + BHT	3^{d}	$9^{e,f}$	14^{e}	25^{e}	34^{e}
Olive r. extd oil + EE layer	3^{d}	3^{d}	10^{d}	18^{d}	29^{d}

[a]Values within a column followed by different superscript letters (d-j) are significantly different ($P < 0.05$).

[b]Butylated hydroxytoluene.

[c]Olive residue extracted oil.

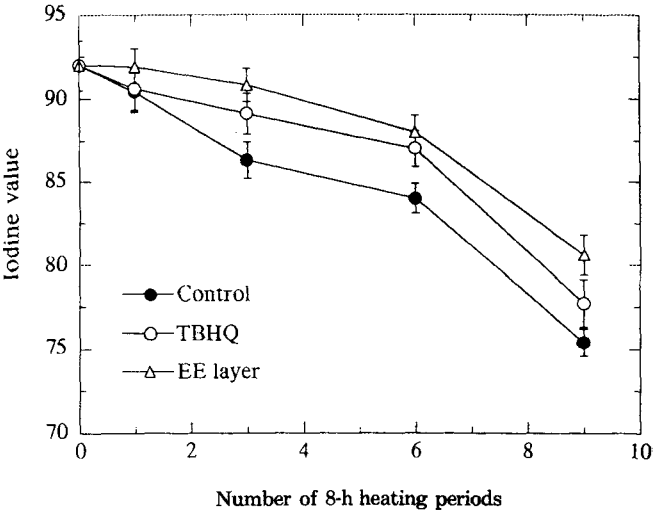


FIG. 2. Effect of tertiary butylhydroquinone (TBHQ) and ethyl ether (EE) layer, at a concentration of 0.05%, on the iodine value of the olive residue extracted oil during frying at 180°C. Data are averages of duplicate trials ± SD (bar).

TABLE 3

Antioxidant Activity of the Ethyl Ether (EE) Layer Added to Vegetable Oils Stored in the Dark at 35°C (concentration 0.01%)[a]

Sample	Peroxide value (meq/kg) after days				
	0	40	70	80	90
Corn oil	2^{b}	$7^{c,d}$	19^{d}	56^{g}	82^{g}
Corn oil + BHT	2^{b}	6^{c}	$17^{c,d}$	29^{e}	40^{e}
Corn oil + TBHQ	2^{b}	2^{b}	8^{b}	$11^{b,c}$	12^{c}
Corn oil + EE layer	2^{b}	$5^{b,c}$	13^{c}	30^{e}	40^{e}
Olive r. extd oil	3^{b}	13^{e}	33^{e}	40^{f}	54^{f}
Olive r. extd oil + BHT	3^{b}	9^{d}	$16^{c,d}$	20^{d}	23^{d}
Olive r. extd oil + TBHQ	3^{b}	6^{c}	6^{b}	8^{b}	8^{b}
Olive r. extd oil + EE layer	3^{b}	$4^{b,c}$	8^{b}	12^{c}	13^{c}

[a]Values within a column followed by different superscript letters are significantly different ($P < 0.05$). Abbreviations as in Table 2, TBHQ, tertiary butylhydroquinone.

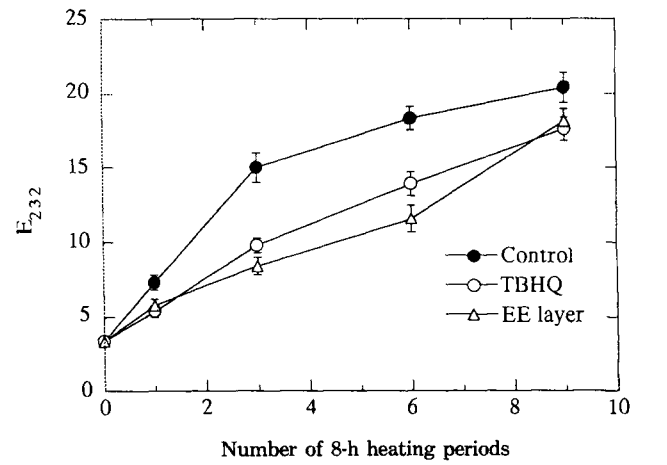


FIG. 3. Effect of tertiary butylhydroquinone (TBHQ) and ethyl ether (EE) layer, at a concentration of 0.05%, on the E_{232} of the olive residue extracted oil during frying at 180°C. Data are averages of duplicate trials ± SD (bar).

When the oils are heated at frying temperature, there is a loss in unsaturation, which accelerates as heating progresses. The IV has been reported to parallel the loss of polyunsaturates in heated vegetable oils (18). Heating also causes the formation of linoleic hydroperoxide and conjugated dienes resulting from its decomposition, which show an absorption band at 232 nm. The IV and E_{232} value of refined olive residue extracted oil with added EE layer or TBHQ are plotted against the number of 8-h heating periods in Figures 2 and 3, respectively. The loss in IV and, consequently, in total unsaturation was depressed by the addition of the antioxidants. The initial sharp rise of the E_{232} value, on the other hand, was reduced in treatments containing either antioxidant. The differences in the IV and in the E_{232} value between the oil samples containing TBHQ or an EE layer were not statistically significant in most cases. Additionally, the RI of the olive residue extracted oil showed a smaller change after nine 8-h periods of heating (*i.e.*, from 1.4702 to 1.4721) when either an EE layer or TBHQ were used, compared to the oil with no additives (from 1.4702 to 1.4736, respectively).

Table 4 presents the analytical data of fatty acid methyl esters of the starting oil before and after nine days of frying. The ratio of 18:2/16:0 is also listed as indicative of the degree of oil deterioration (19). The EE layer and TBHQ resulted in the same amount of remaining 18:2, which was higher than that of the oil with no additives. This means the unsaturated fatty acids were equally protected by both antioxidants. The 18:2/16:0 value was also increased by the addition of either antioxidant, thus showing a lower deterioration degree. Therefore, the EE layer contributed to the stability of refined olive residue extracted oil at frying temperature, its efficiency being the same as that of TBHQ.

Analysis of the EE layer. Preliminary TLC analysis of the EE layer was conducted on silica gel plates (5715

TABLE 4

Fatty Acid Methyl Esters of Olive Residue Extracted Oil Before and After Nine Days of Frying at 180°C

Treatment	16:0	18:0	18:1	18:2	18:2/16:0
Day 0					
Fresh oil	12.2	1.9	70.5	13.7	1.12
Day 9					
No additive	15.4	2.8	74.1	6.3	0.40
TBHQ[a]	14.7	2.5	73.3	8.4	0.57
EE layer[b]	14.2	2.3	73.4	8.4	0.59

[a]Tertiary butylhydroquinone.

[b]Ethyl ether layer.

Merck) with 10% methanol in dichloromethane. A poor resolution was obtained, but the reaction of most of the bands to aluminum chloride and to naturstoffreagent showed the presence of flavonoids.

The fractionation of the extract on a silica gel column (Fig. 1) gave six fractions, which were tested on lard. The results are given in Table 5. Fraction Fr2 showed the highest activity, equal to that of the EE extract. Fr2 was refractionated on a silica gel column (Fig. 1). Subfraction 2B was developed on silica gel plates in CAF. Three bands with R_f 0.30, 0.54 and 0.58, namely A, B and C, respectively (Fig. 1), had a positive response to β-carotene spray and a yellow color when sprayed with aluminum chloride, which is characteristic of flavonoids (11,12).

Band A (R_f 0.30) was developed on cellulose plates with 15% acetic acid and gave a band at R_f 0.53 that showed a positive reaction to the β-carotene spray. Observation under UV showed a dark color that persisted after exposure to ammonia fumes and is characteristic of flavones, dihydroflavonols and some flavanones. Spraying with naturstoffreagent resulted in an orange-yellow fluorescence, which indicates the presence of quercetin derivatives (13). Two-dimensional analysis on cellulose plates with 15% acetic acid and BAW gave single spots at R_f 0.53 and 0.75, respectively, suggesting that the band was chromatographically pure. Cochromatography in the same systems with reference standards indicated that the compound was the dihydroflavonol dihydroquercetin (taxifolin). Its chemical structure, as presented by Das and Pereira (7), is indicated in Table 6. This was further confirmed by spectral analysis. The methanol and sodium methoxide spectra of the eluant of this band were the same as those of the dihydroquercetin standard (obtained from Sigma Chemical Co., St. Louis, MO). References on isolation of antioxidants from plants claim that dihydroquercetin is a highly effective antioxidant (4,6). Also, a high antioxidant efficiency has been reported for commercial preparations of dihydroquercetin and quercetin (5,7).

TLC analysis of band B (R_f 0.54) on polyamide sheets with BMM as the developing system gave two bands with R_f 0.50 and 0.59. A positive response to β-carotene indicated that both these bands possessed antioxidant activity. Both bands had the characteristic yellow color of flavonoids when sprayed with aluminum chloride (11,12). Observation of the chromatograms under UV showed a dark color that persisted after exposure to ammonia fumes. After spraying with naturstoffreagent, the band with R_f 0.50 had a light red fluorescence, which is characteristic of eriodictiol (20), while the band with R_f 0.59 had a bright, green-yellow color, which indicates the presence of 4′-OH flavonoids, especially kaempferol derivatives (12,13).

Two-dimensional chromatography on cellulose plates with BAW and 15% acetic acid was used to test the purity and to characterize the compounds of each one of the bands. The band with R_f 0.50 gave a single spot at R_f 0.90 with BAW and at R_f 0.27 with 15% acetic acid. Cochromatography on cellulose plates in two solvent systems (BAW and 15% acetic acid) with reference standards indicated that the compound was eriodictyol. The band with R_f 0.59 gave a single spot at R_f 0.79 with BAW and at R_f 0.46 with 15% acetic acid.

Further identification of the antioxidants was attempted by spectral analysis. The methanol, sodium methoxide, sodium acetate, sodium acetate-boric acid, aluminum chloride and hydrochloric acid-aluminum chloride spectra were recorded. The eluant of the band with R_f

TABLE 5

Antioxidant Activity of the Fractions Obtained from the Ethyl Ether (EE) Layer Tested in the Dark on Lard at 65°C (concentration 0.02%)[a]

	Peroxide value (meq/kg) after days[b]				
Additive	3	6	10	13	16
None	5[d,e]	13[f]	210[f]		
EE layer	3[d,e]	4[d]	6[d]	6[d]	8[d]
Fr1[c]	2[d]	5[d,e]	10[e]	20[f]	
Fr2	3[d,e]	4[d]	5[d]	6[d]	8[d]
Fr3	3[d,e]	4[d]	5[d]	6[d]	17[f]
Fr4	5[d,e]	8[e]	10[e]	11[e]	19[f]
Fr5	6[e]	30[g]			
Fr6	5[d,e]	28[g]			
Butylated hydroxyanisole	3[d,e]	4[d]	5[d]	6[d]	12[e]
Butylated hydroxytoluene	3[d,e]	3[d]	5[d]	6[d]	8[d]

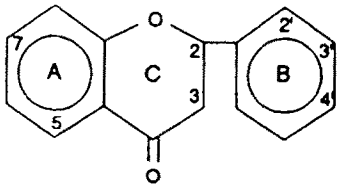
[a]Values within a column followed by different superscript letters (d–g) are significantly different ($P < 0.05$).

[b]Peroxide values of all samples at day 0 was 1.

[c]Fractions eluted by column chromatography of the EE layer as defined in Figure 1.

TABLE 6

Chemical Structure of the Flavonoids Isolated from Oregano Leaves



	Substituent positions				
	Ring A		Ring C	Ring B	
Compound	5	7	3	3′	4′
Apigenin (flavone)	OH	OH	H	H	OH
Eriodictyol (flavanone)	OH	OH	H, H	OH	OH
Dihydroquercetin (dihydroflavonol)	OH	OH	H, OH	OH	OH
Dihydrokaemferol (dihydroflavonol)	OH	OH	H, OH	H	OH

0.50 gave spectra identical to those of standard eriodictyol (obtained from the laboratory of Pharmacognocy, University of Athens, Greece). The absorption maxima of the eluant of the band with R_f 0.59 were identical to those given by Mabry (17) for the dihydroflavonol dihydrokaempferol. The chemical structure of both compounds is indicated in Table 6.

The antioxidant activity of eriodictyol has been reported by Hudson and Lewis (5). To our knowledge, the antioxidant activity of dihydrokaempferol has not been examined, but the flavonol kaempferol has been reported as a highly effective antioxidant (3,7). Previous experimental results showed that dihydroflavonols had the same antioxidant activity as the corresponding flavonols, suggesting either that the 2,3 double bond is not of major importance to antioxidant activity, or that conversion of dihydroflavonols to flavonols took place while the compounds were in contact with the oxidizing lipids and that the conversion might account for the antioxidant activity (6).

Observation of band C (R_f 0.58) under UV showed a dark color that persisted after exposure to ammonia fumes. Also, a green-yellow color appeared after spraying with naturstoffreagent. These reactions indicated the presence of a flavone, dihydroflavonol or flavanone with a 4′-OH group (12). Cochromatography with three solvent systems, *i.e.,* EAW on cellulose plates, CAF on silica gel plates and MAW on polyamide sheets, with reference standards indicated that the band with R_f 0.58 was chromatographically pure and coincided with the flavone apigenin.

Spectral analysis of this band and of apigenin standard (obtained from Fluka Chemika-Biochemika, Buchs, Switzerland) showed that the methanol, sodium methoxide, sodium acetate, sodium acetate-boric acid, aluminum chloride and hydrochloric acid-aluminum chloride spectra of both compounds were identical. The chemical structure of apigenin is indicated in Table 6. Apigenin has been examined as an antioxidant by other investigators (7,21), though its activity seemed to be much lower than in our experiments.

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